Author's response to reviews

Title: Glioma stem cells are more aggressive in the recurrent tumor with malignancy progression than in the primary tumor and they both can be maintained for long-term in vitro

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Author's response to reviews:

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Dear Editors,

Enclosed please find our manuscript entitled “Glioma stem cells are more aggressive in the recurrent tumor with malignancy progression than in the primary tumor and they both can be maintained for long-term in vitro” for consideration of publication in BMC Cancer.

Our manuscript has been revised according to the comments of both the editors and two referees.

Every questions and revision are listed in detail as below:

Questions of the editors:

1. Question: Please note that we are concerned about whether your submission represents an advance on the literature. We would invite you to consider this point carefully when revising your manuscript.

Answer: More importantly, we demonstrated that the tumor cells expressing glioma stem cell marker CD133 were preserved both in vitro and in vivo even during serial passage and in vivo subtransplantations for almost four years. GSCs were actually at their primitive stage of differentiation with low activity of autophagy, to the best of our knowledge, the first report about autophagy of GSCs. Our data also represent the first report to demonstrate that CD133+ GSCs...
of primary and recurrent counterpart with malignancy progression harbor different genetic abbreviations, which help to explain why the latter showed more aggressive biological behaviors.

2. Question: Please re-write/format your abstract to the BMC style.

Answer: The abstract has been re-write in the manuscript according to the BMC styles.

3. Question: Informed consent must also be documented.

Answer: The original informed consent were obtained twice both for the primary and recurrent tumor of the same patient before surgery. The original informed consent (in Chinese) and the English translation were presented as affiliated files.

4. Question: We recommend that you ask a native English speaking colleague to help you copyedit the paper.

Answer: The manuscript has been modified by a native English speaking expert to minimize the grammatical errors and other language mistakes.

5. Question: Please include the following statements in your revised manuscript: Competing interests.

Answer: The statements of competing interests of the manuscript have been supplemented in the relevant place of the manuscript.

6. Question: Authors’ contributions - Please include an Authors’ contributions section before the Acknowledgements and Reference list.

Answer: Authors' contributions section has been added before the acknowledgements and Reference list.

Questions by John Ohlfest

1. Question: The title is still not grammatically correct. The words “for long-terms” should be changed to read “long-term”. First sentence in intro, change “malignance” to “malignancy”. The paper is full of misspellings that should be corrected. The paper is full of additional grammatical errors that should be corrected.

Answer: We invited a native English speaking expert to read the whole manuscript carefully for the purpose of minimizing the grammatical errors and other language mistakes.

2. Question: The authors should state what criteria they use in order to refer to a cell as a “glioma stem cell”. This should be clearly explained in the introduction of the paper. They attempt to define glioma stem cells in the first sentence of the discussion, but omit tumor initiation from this description, which is probably the most important criteria.
Answer: The complete criterion of glioma stem cells were fully explained in the introduction of the manuscript.

3. Question: Glioma stem cells should be defined based on their capacity to self renew, differentiate (which the cells in the current study failed to do), and tumor initiate.

Answer: More results were added in the 3rd paragraph of the results of this paper which described the results of in vitro differentiation of GSCs in detail.

4. Question: The expression of CD133 should not be listed as a requirement since several studies have identified CD133- glioma stem cells:

CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res. 2007 May 1;67(9):4010-5.


Answer: Although CD133, a 120 kDa cell-surface protein that is a marker of normal human neural stem cells, is not a specific marker or a golden criteria for identification of glioma stem cells, it was really a very important marker and has been widely used for the enrichment of tumor stem-like cells from brain tumors in majority of relevant studies. Just in the two reference papers the reviewer mentioned above, CD133 were also used necessarily to subdivide the different cell populations. As we know, not only tumor stem cells can form tumor, the tumor progenitor cells also has the capacity to form tumora few of the latter can retro-differentiate into tumor stem cells to re-establish the tumor. So according to the two reference papers the reviewer mentioned above, there is no enough evidence to prove whether the so called CD1133- tumor stem cells are really tumor stem cells, or CD133- tumor progenitor cells. So it’s easy to understand the data in the 2 papers, i.e. the sphere-forming ration of a single cell from CD133- sphere was 0.5-2%, while the counterpart from CD133+ sphere was higher, namely 2-5%, indicating the self-renewal capacity of so called CD133- stem cells was lower than that of CD133+ stem cells.

5. Question: The authors do cite the cancer research paper in the discussion but state the data incorrectly. That is, autologous CD133+ and CD133- cells were not compared for tumor initiation in that paper.

Answer: This paper was read carefully again, and the citation of the relevant data in the discussion part was modified as follows:

Beier’s studies revealed four of 15 cell lines derived from primary glioblastomas grew adherently in vitro and were driven by CD133- tumor cells that fulfilled stem cell criteria. Both CD133+ and CD133- subtypes of GSCs were similarly tumorigenic in nude mice in vivo[19], indicating CD133 expression is not enough for identification of GSCs, more efforts should be made to find the specific GSCs
marker. Up to now, though the functional criteria of GSCs are sophisticated and inconvenient to perform, it should not be neglect.

6. Question: Glioblastoma multiforme is misspelled as “multiform” in the first paragraph of the methods. This is just one of many spelling errors throughout the paper.

Answer: we have corrected this misspelling, and checked the whole manuscript to minimize such kind of error.

7. Question: Fig3, the data should be shown as a histogram where the isotype control is overlayed on the CD133 stained sample.

Answer: Fig3C and Fig3F have been modified to be shown as a histogram where the isotype control is overlayed on the CD133 stained sample.

8. Question: The localization of CD133 staining shown in Fig 2 is not membranous (except for Fig2B), and therefore, probably just non-specific binding of the antibody because CD133 is a membrane bound protein. Moreover, the figure legend corresponding to figure 2 does not match the text where expression of GFAP and TuJ1 is referred to.

Answer: The atypical pictures in Fig2A-C were changed to the proper images which showed membranous staining of CD133. The figure legend corresponding to figure 2 was rewrite.

9. Question: Figure 4 is not sufficient to show what the authors describe in the text. Figure 4b-d should be shown at 1-2X magnification so that the degree of infiltration in relation to the inoculation site is visible. As it is currently, the images are at too high of power to determine anything about invasion into the contralateral side.

Answer: Yes, Fig4C and 4D were changed according to the reviewer’s opinion. Thus xenograft tumor for SU-1 cells (Fig 4A) and xenograft tumor for SU-2 cells(Fig4C) could be shown at 1-2X magnification so that the degree of infiltration in relation to the inoculation site is visible. Fig4B and 4D were the local magnification of Fig4A and 4C, respectively.

10. Question: The authors make a repeated effort to say that nobody has established a long-term glioma stem cell line. This is simply not true. Most importantly is the recent paper listed below where they established a series of glioma stem cell lines. Therefore, there is really nothing novel about the current paper.

Glioblastoma-derived stem cell-enriched cultures form distinct subgroups according to molecular and phenotypic criteria. Oncogene. 2008 May 1;27(20):2897-909. Answer: We upload our manuscript to BMC Cancer in April 2008, the reference paper which the reviewer offered was published in May this year. So we cannot update the information in time, and there is very few papers focused on this point till now. However, we will update the information in the
Question by Joerg Wischhusen

1. Question: The manuscript requires some language editing. Even though the authors manage to communicate their message quite clearly, there are too many unidiomatic phrasings, awkward sentence constructions, typing errors and other mistakes that should be corrected.

Answer: This paper was read carefully to correct grammatical or spelling mistakes, we also invited a native English speaking expert to read the whole manuscript carefully for the purpose of minimizing the grammatical errors and other language mistakes.

2. Question: There is no indication of the treatment the patient received after surgery. This information has to be supplied – and it may also have implications on the interpretation of the data. Radiotherapy, for example, is known to result in a relative enrichment of stem cell-like glioma cells (Bao et al., Nature. 2006 Dec 7;444(7120):756-60.). Assuming that the patient has received standard treatment including post-surgical radiotherapy, the increased proportion of CD133+ cells in the recurrent tumor could thus be due to this treatment.

Answer: Information about the adjunctive treatments the patient received after surgery was added in the 1st paragraph of materials and methods part. Malignant progression and enrichment of GSCs may be the results of several aspects, including intrinsic accumulation of genetic abbreviation of tumor, radiotherapy, etc., our data suggest not only the quantity of the GSCs increased, but also the quality of the GSCs changed along with tumor malignancy progression and recurrence. Up to now, there is no enough proof to make a conclusion that the radiotherapy is the only responsible factor.

3. Question: The characterization of the glioma stem cells is rather incomplete. Thus, the data from the experiments that confirmed the self-renewal capacity should be presented. (What is the percentage of sphere-forming cells after dissemination into single cells? Does this percentage remain constant over various cycles?)
Answer: The data from the experiments that confirmed the self-renewal capacity were presented in the first paragraph of the results of the manuscript.

4. Question: Finally, the authors injected 200,000 cells into NC nude mice. Normally, much lower cell numbers should be sufficient if the cells were really tumor stem cells. In addition, such an experiment could give an idea, which proportion of the cells in these “GSC lines” really display tumor stem cell features. Thus, this information would be a required before the cell lines can really be considered GSC lines.

Answer: First, we inoculate the nude mice with the cells dissociated from the tumor spheres under stem cell culture condition, in which should contained CD133+ tumor stem cells, tumor progenitor cells, and some differentiated tumor end cells. Second, pure CD133+ glioma stem cell line is not available now, we cannot stop completely the proliferation and differentiation of these tumor stem cells in vitro even in the culture condition favoring stem cell growth. The percentage of CD133+ cells in these cell lines varied widely. Third, in some literatures, 102-104 CD133+ tumor stem cells can form tumor in NOD-SCID mice with both T and B lymphocytes deficiency; while others reported 104-106 stem cells can form tumor in nude mice with only T cell deficiency:

CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles. Cancer Res. 2007 May 1;67(9):4010-5.


5. Question: In Figure 3, it is not clear, what is shown in the inset. Is this the isotype control? In addition, it is very hard to depict a CD133-positive population in this FACS staining.

Answer: The inset in Fig3C, 3F is the isotype control, and now they have been modified to be shown as a histogram where the isotype control is overlayed on the CD133 stained sample.

6. Question: Obviously, the glioma stem cells only form a subpopulation of the cultured cells. Thus, it is doubtful whether the cell shown in Fig. 5 is really a GSC or not. It is not even clear whether this cell belongs to the CDD133+ subpopulation in the culture or was just picked at random. This should at least be discussed. In addition, the authors should be careful with the use of the term “glioma stem cells”. While it appears that they have established cell lines containing GSC, the actual GSC will only constitute a small fraction of the cell lines.

Answer: CD133+ GSCs were observed under the transmission electronic microscope. The necessary information about how to purify the CD133+ GSCs was supplemented in the relevant paragraph introducing transmission electronic
microscopy of the materials and methods section.

7. Question: In Figure 6C, error bars are lacking. Moreover, GAPDH is a poor reference gene when tumor cells are being compared with cultured normal human astrocytes. Due to the different metabolic activity, GAPDH expression may be hugely different between those two cell types. Thus, the data need to be confirmed using a further internal standard (like 18 S rRNA) or a combination of markers (Thelin et al., J Biotechnol 1999, 75:291-295.). This could also help to answer the question whether the very slight downregulation of Pten mRNA is statistically significant. In addition, some information on the controls and standards used should be added to the legend to Figure 6.

Answer: In Figure 6C, error bars have been added, and the reference gene was changed to #-actin when real-time PCR was performed again.

Yours truly,

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